P16INK4a Overexpression in Cervical Biopsies Collected from Women with Normal and Equivocal Pap Smears

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Abstract:

Background: Cervical cancer is the third most common cancer among women worldwide Immunohistochemical expression of p16INK4a has been relatively well established in cervical biopsy specimens.

Objectives: Our hypothesis is to evaluate the usefulness of p16 immunostaining on tissue sections in borderline cases.

Methods: This is a case-control study with a total of cervical samples from 60 women (25-66 years age) divided into three groups (I, II and III). The study was conducted at the Colposcopy Clinic in Baghdad Teaching Hospital – Medical City Complex, during the period from June 2013 through July 2014. The samples were analyzed by cytopathology, histopathology and p16INK4a immunostaining.

Results: Group I showed negative p16 reaction. Among group II; one (5%) showed mild positive, 9 (45%) moderate positive and 10 (50%) high positive reactions. Group III specimens showed p16 positivity.

Conclusion: p16INK4a immunohistochemistry is one of the best candidates for histologically indeterminate lesions.

Key words: p16INK4, cervical cancer, human papillomavirus, cervical intraepithelial neoplasia

Introduction:

Cervical cancer is the third most common cancer among women worldwide. It affects about 15 and kills about 8 women per 100,000 per year. In 2008, cervical cancer was responsible for 275,000 deaths worldwide. Currently, almost 85% of cases occur in developing countries [1, 2]. HPV DNA presence has been demonstrated in clinically symptom-free mucosal and epidermal sites of the cervix, the skin, or the larynx [3]. Recent evidence suggested that almost 100% of cervical cancer is attributable to HPV infection [4].

Monitoring women using cervical smear testing (Pap test) has drastically reduced the incidence of cervical cancer in countries with an organized screening programmes [5]. The clinical management of preinvasive cervical lesions relies on histological examination to confirm cervical intraepithelial neoplasia (CIN) and it's grading [6]. Previous studies have demonstrated that the histologic detection and grading of HPV-induced CIN, especially the low-grade categories such as atypical squamous metaplasia, koilocytosis, and CIN1, have poor reproducibility and are limited by interobserver variability [7, 8, 9, 10, 11]. Therefore, there is a need in pathology practice for a biomarker that will help in distinguishing true dysplasia from dysplasia mimics [12]. Immunohistochemical expression of p16 ^{INK4a} has been relatively well established in cervical biopsy specimens with excellent inter- and intraobserver reproducibility [11, 13]. The overexpression of this marker has been demonstrated in cervical cancers as a result of functional inactivation of pRb by the HPV E7 protein [14, 15, 16].

Our hypothesis is to evaluate the usefulness of p16 immunostaining on tissue sections in borderline cases. This could subcategorize atypical squamous cells of undetermined significance (ASCUS) and low-grade squamous intraepithelial lesion (LSIL) (cervical intraepithelial neoplasia CIN1) into cases with and without dysplasia. This approach would avoid invasive interventions and prevent potentially increased morbidities. Developing and adopting this test may support early detection, and hence, lengthening of follow-up interval

Materials and methods:

In this study, p16 protein has been demonstrated in formalin-fixed paraffin-embedded (FFPE) cervical samples. The target population was ever-married women attending the Colposcopy Clinic in Baghdad Teaching Hospital – Medical City Complex, during the period from June 2013 through July 2014.

This is a case-control study with a total of 60 women (25-66 years age). They were referred to the clinic from general hospitals and primary health care centers. Pregnant women, bleeding or menstruating at time of test, women with previous surgery of the cervix, e.g. conization or total hysterectomy, women with other pelvic or adnexal diseases or masses, with any other chronic disease or malignancy outside the pelvic organs, and women under immunosuppressant therapy *or* (HIV) infection were excluded.

The purpose; including questionnaire and investigations, were fully explained to all participants and a verbal consent was obtained. The enrolled women were divided into three groups; group I, twenty women with healthy looking cervices and normal Pap smears. Group II, twenty women with unhealthy looking cervices and abnormal Pap smears, presented either with history of vaginal discharge unresponsive to treatment, lower abdominal pain, low back pain, and postcoital bleeding, or combination of two or more symptoms. Vaginal discharge was present among the majority of patients. The third group (group III, twenty

women): were well known cases of invasive cervical cancer, presented mainly with postcoital and/or postmenopausal bleeding, offensive blood-stained vaginal discharge and abdominal and back pain. On examination, red ulcerated surface, bleeding on contact or fungating lesions were observed which were diagnosed as cervical cancer. Hysterectomy was performed according to the cancer stage and the diagnosis was confirmed histopathologically as squamous cell carcinoma. The specimens taken from this group were considered as the positive controls for this study.

A full medical, surgical and gynecological history was obtained from each candidate with special attention paid for age, occupation, education, last menstrual period (LMP), intermenstrual bleeding, postcoital bleeding, age of marriage (sexual debut), parity, type of delivery (normal vaginal delivery (NVD)/ Caesarean Section (CS), abortions, vaginal discharge, combined oral contraceptive pills (COCP), intrauterine contraceptive device (IUCD), smoking and alcohol consumption

Pelvic examination was performed for adnexal masses or tenderness, prolapse, cervical polyps, warts and cervical discharge were noted and evaluated. A speculum inserted for the visualization of the cervix and for sample collection. Samples obtained in this study were; conventional Pap smear, liquid-base cytology, and cervical punch biopsies.

Liquid-base cytology method; A broom-like cervical collection device was inserted into the endo and ecto cervix then transferred to the ThinPrep PreservCyt® vial containing methanol based, buffered preservative solution, Hologic Inc. USA. Thin Prep vials were located into ThinPrep 2000 automated processor for the transfer of cells from the vial to the slide. Conventional Pap test was done afterwards using Ayer's wooden spatula and the scraps was fixed onto labelled glass slides and preserved in 96% ethyl alcohol-containing vial as a fixative. The slides were then stained with Iraqi Pap stain modified by Al-Rawi, 2002. Cytologic diagnosis was made according to the cervical cytology criteria of the 2001 Bethesda System. The cervix was inspected with a binocular colposcope (Carl Zeiss, Germany 2009), (Visual Inspection with Acetic Acid was performed (VIA). Then logul's iodine (Schiller's iodine) was applied. Punch biopsy (3-5 mm) size was taken from key sites of acetowhite epithelium, the Schiller positive regions (not stained dark brown/black), immediately fixed in 10% buffered neutral formalin for histopathology. When no acetowhite area was observed, a biopsy was obtained at the 12 o'clock position near the squamocolumnar junction. The fixed samples were consequently embedded in paraffin. Formalin-fixed paraffin-embedded (FFPE) blocks were sectioned into 5 µm thick serial sections, using a Histoline semiautomatic microtome. Sections were mounted on positive-charged slides and left to air dry and one of each section was mounted on an ordinary slide and used for Haematoxylin & Eosin staining. For the detection of p16INK4a protein expression, Immunohistochemistry (IHC) was performed using anti p16 (F-12): sc-1661 monoclonal antibody and the detection kit used was the standard one recommended by the same manufacturer for antibodies: ImmunoCruz mouse LSAB Staining System: sc-2050 (Santa Cruz-USted A) (Immunocruz TM staining system using horseradish peroxidase (HRP)-streptavidin complex. The sections were dewaxed in xylene, rehydrated in a series of gradient alcohol solutions and rinsed in distilled water DW. Sufficient amount of 0.3% hydrogen peroxide block was added to cover each tissue section in order to block endogenous peroxidase activity, and the slides were incubated in a humid chamber for 20 minutes at 37°c. Slides then were washed with PBS three times, three minutes each. Slide were drained and blotted to get rid of the excess PBS; avoiding the dryness of the slides between steps. The primary antibody (anti-p16 (F-12) monoclonal antibody/ Santa Cruz- USA) was applied and the slides were incubated overnight at 37 ^{o}C, washed 3 times in DW/Tween 20. The sections were sequentially incubated with secondary antibody (Mouse Specifying Reagent unconjugated) for 30 minutes at room temperature. Goat anti-rabbit HRP conjugate was applied and the slides were incubated for 45 minutes at 37 ^{o}C, rinsed twice in DW/Tween 20. A drop of freshly prepared DAB was added to each section and incubated for 4-10 minutes, rinsed thoroughly in DW, counter-stained with Harris's Hematoxylin, dehydrated in ascending grades of ethanol, cleared in xylene, mounted in DPX and covered with cover slips. p16INK4a immunoexpression was evaluated according to parameters set forth by Klaes. Positive control tissues for the test probe were the diagnosed cases of cervical cancer while the negative controls were normal cervical specimens with each run. P16 overexpression or a positive reaction showed brownish deposition in the cytoplasm of the HPV infected cells. The test included evaluation of the percentage of cells staining positively; 1) Negative; sporadic (and/or no staining), 2) Patchy; 5% of cells positive, 3) Mild, moderate; 5%–25% of cells positive, 4) High; 25% of cells positive) [5,8,9]. The distribution of staining within the epithelium was evaluated (lower third, one third to two thirds, and two thirds to complete thickness) [5,8]. The tissue sections were photographed using Canon digital camera (model EOS Kiss X6i, resolution power of 16 mega pixel). Based on criteria of cytology, histopathology, and P16 staining reactions, the mean, median and standard deviation were calculated. Chi-square test was used to detect the significance between variables. All statistical data analyses were done using SPSS program (version-

20). P-value was considered significant when <0.05 and highly significant when it was <0.01

Results:

General characteristics (age, age of marriage or sexual debut and parity) of all groups (I, II and III) of women included in this study are summarized in tables 1, 2, 3 respectively. The age range of the total number of all the groups in the study was 25 to 66 years with a mean of 42.8 years, a median of 45 years and standard deviation of ±9.509674. The ages of women in group I with normal looking cervices and normal Pap smear results ranged from 31-51 years with a mean age of 39.5, median of 40 with standard deviation of ±6.270. In group II women with abnormal looking cervices and abnormal Pap smear results, the ages ranged from 25-53 years with a mean age of 37.35, a median of 35 with standard deviation of ±7.895. While the ages of women in group III ranged from 47-66 years with a mean age of 51.7, a median of 51.5 with standard deviation of ±4.680 (Table 1).

Chi square test was done to compare group results concerning ISH & P 16 among various groups and showed a highly significant association (P= 0.00). And by the application of independent sample t-test to compare the effect of age among groups with other variables, age distribution was highly significant among all age groups; GI &GII (p=0.00), GI & GIII (p=0.00), GII &GIII (p=0.00) in both ISH and p16 test results

The age of marriage of group I ranged from 13-26 years of age with a mean of 20.4 and a median of 20.5 and standard deviation ±4.235. The age of marriage within group II ranged from 14-40 years with a mean of 20.25 and a median of 18 and standard deviation ±6.163. In group III, the age of marriage ranged from 14-28 years with a mean of 19.1 and a median of 18 and standard deviation ±3.625 (Table 2). By the application of independent sample t-test to compare the effect of age of marriage among groups. The age of marriage was not significant, GI & GII (p=0.93), GI & GIII (p=0.12), GII & GIII (p=0.30)

In group I, the parity (full-term babies) ranged between (0–9) births with a mean of 4.25, a median of 4 and standard deviation of ±2.314. Among group II, the parity (full-term babies) ranged between (0–8) births with a mean of 4.2, a median of 4 and standard deviation of ±1.880. Among group III, the parity ranged between 1–8 births with a mean of 4.05, a median of 4 and standard deviation of ±1.761 (Table 3).

By application of independent sample t-test to compare the effect of parity among groups with other variables, the distribution was insignificant among all groups; GI & GII (p= 0.66), GI & GIII (p= 0.82), GII & GIII (p= 0.79)

Pap test results: The Pap test results for Group I: All patients had negative Pap test or negative for intraepithelial lesion or neoplasia NILM compared to GII (p=0.001). In group II: 5 (25%) of the samples were negative for intraepithelial lesion or malignancy NILM, 7 (35%) of the samples were diagnosed as low grade squamous intraepithelial lesion or mild atypia. The remaining 8 (40%) showed atypical squamous cells of undetermined significance ASC-US. Group III women were presenting with fungating lesions and were diagnosed cases of invasive cervical cancer, therefore, Pap test was not done (Figure 1, Table 4)

Histopathology Test Results: Histopathology Test results for group I: Normal histology, cervicitis, no atypia compared to GII (p=0.009). Group II test results: 4 (20%) of the tissue sections showed cervicitis or reparative atypia, 6 (30%) of the sections showed mild atypia or basal cell hyperplasia, and the remaining 10 (50%) were diagnosed as CIN1 with koilocytosis, parakeratosis and acanthosis. For group III: Samples were collected from the patients after hysterectomy, they were diagnosed as invasive squamous cell carcinoma, and high mitotic activity was observed. (Figure 2, Table 5).

p16INK4a Test Results: The test results for Group I: All patients had negative p16 reaction compared to GII (p=0.000). Among group II; one sample (5%) showed mild positive reaction with staining in focal sporadic areas (Figures 4, 5). A moderately positive staining reaction in the basal cells (lower third) of the epithelium among 9 (45%) samples (Figures 6, 7). 10 samples showed high positive reaction extending throughout the lower two thirds of the epithelium (Figure 3, Table 6). Among group III specimens, all the cells of the epithelium were stained with p16 with stromal invasion. Chi square was done to compare group results concerning P 16 among various groups, it was highly significant (p= 0.001)

Table 1: The age distribution among the three groups I, II, and III

Age (years)	No. of women Group I (%)	No. of women Group II (%)	No. of women Group III (%)
25-29		2 (10)	
30-34	6 (30)	7 (35)	
35-39	3 (15)	3 (15)	
40-44	6 (30)	3 (15)	
45-49	4 (20)	3 (15)	9 (45)
50-54	1 (5)	2 (10)	5 (25)
55-59			5 (25)
>60			1 (5)

Table 2: The age of marriage (sexual debut) among the three groups I, II, and III

Age (years)	No. of women Group I (%)	No. of women Group II (%)	No. of women Group III (%)
<15	1 (5)	2 (10)	1 (5)
15-19	13 (65)	10 (50)	13 (65)
20-24	3 (15)	4 (20)	3 (15)
25-29	3 (15)	2 (10)	3 (15)
30-34		1 (5)	
35-39			
40-44		1 (5)	

Table 3: The number of births among groups I, II, and III

Number of live births	No. of women Group I (%)	No. of women Group II (%)	No. of women Group III (%)
---	1 (5)	1 (5)	------
1	2 (10)	1 (5)	2 (10)
2	--------	1 (5)	1(5)
3	4 (20)	3 (15)	4 (20)
4	5 (25)	5 (25)	7 (35)
5	3 (15)	4 (20)	1 (5)
6	3 (15)	4 (20)	4 (20)
8	-------	1 (5)	1 (5)
9	2 (10)	-------	-------

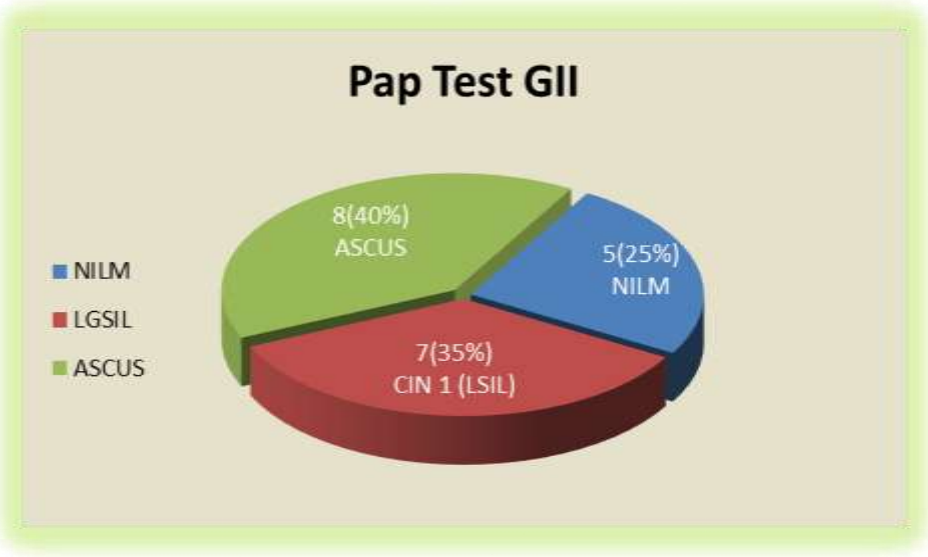

Figure 1: Pie chart showing the Pap test results among group II

Table 4: The Pap test results among the three groups GI, GII and GIII. Pap test was not performed to group III

Pap Test	No. of women Group I (%)	No. of women Group II (%)	No. of women Group III (%)
Negative/NILM	20(100)	5(25)	----
LGSIL (CIN1)	----	7(35)	----
ASCUS	----	8(40)	----
Ca	----	----	----
	20	20	

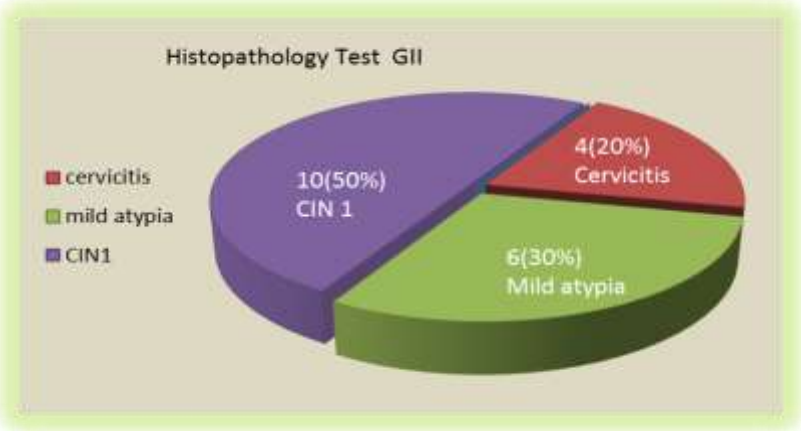

Figure 2: Pie chart showing the histopathology results among group II

Table 5: The histopatholy test results among the three groups GI, GII and GIII

Histopathology	No. of women Group I (%)	No. of women Group II (%)	No. of women Group III (%)
Negative /NILM	20	----	----
Cervicitis	----	4(20)	----
Mild atypia	----	6(30)	----
CIN1, koilocytes, acanthosis, parakeratosis	----	10(50)	----
Ca	----	----	20

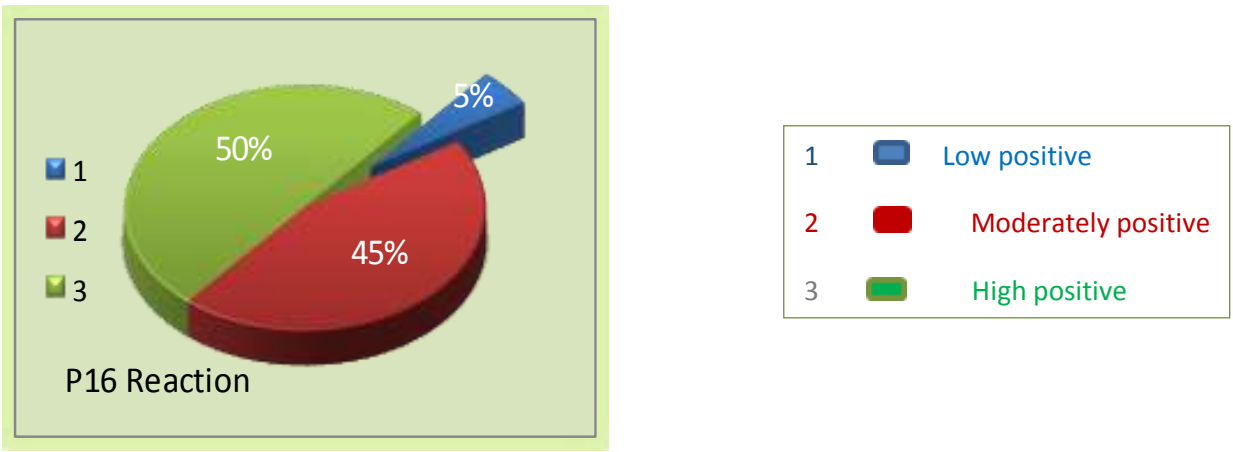

Figure 3: The p16 Immunohistochemistry (IHC) among group II

Table 6: The p16 test results among all the three groups GI, GII and GIII

P16	No. of women Group I (%)	No. of women Group II (%)	No. of women Group III (%)
Negative	20	----	----
Very low (focal areas)	----	1(5)	----
Moderate; Basal cells of epithelium	----	9(45)	----
High; 2/3rds of epithelium	----	10(50)	----
Very High (SCC)	----	----	20

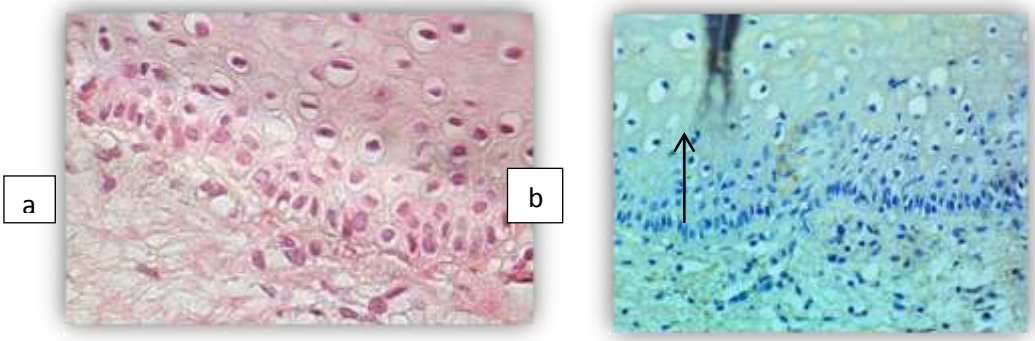

Figure 4 a. Cervical specimen of cervicitis without definite atypia H&E x40, b. p16 immunostaining of the same specimen showing focal, patchy brown staining pattern within the cytoplasm (black arrow) x20

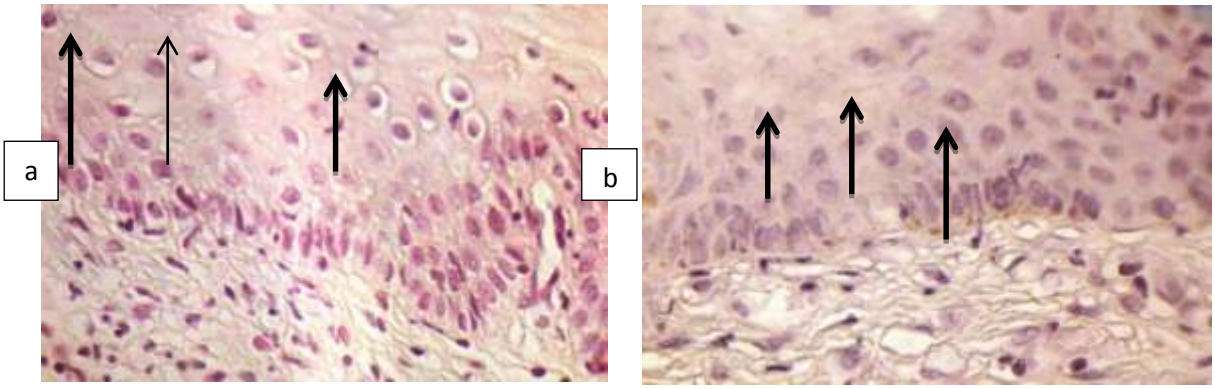

Figure 5: a. Cervical biopsy showing LGSIL with koilocytes (black arrows) H&E x40, b. Immunohistochemical p16 reaction of the same cervical specimen; Mild positive reaction in the basal epithelium indicated by the brown deposits in the cytoplasm (black arrows) X40

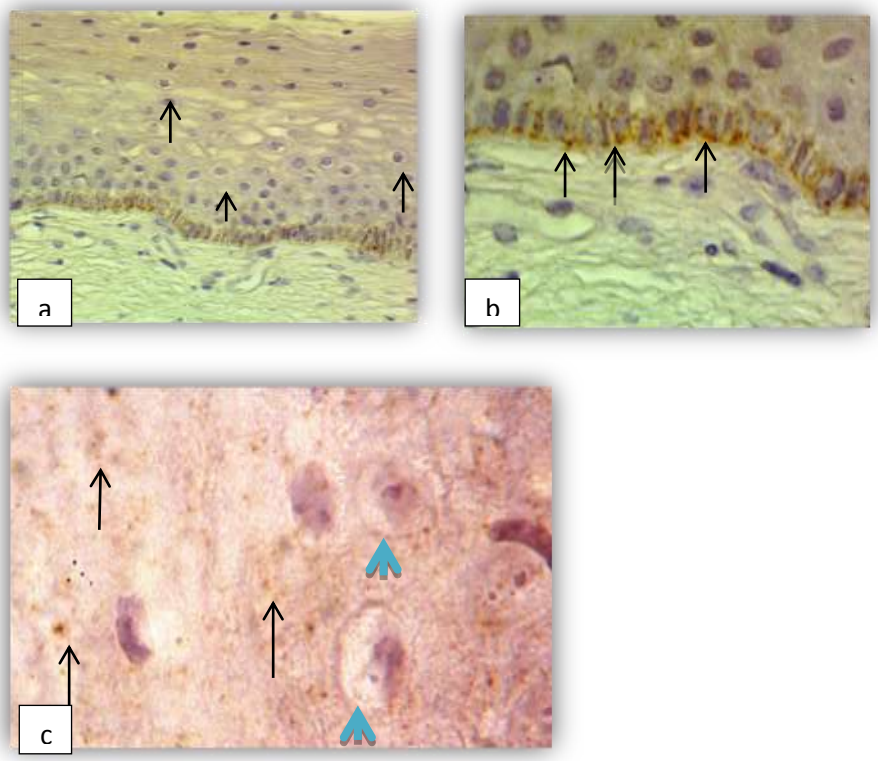

Figure 6: a. Moderate positive p16 overexpression in the lower third of the epithelium. Koilocytes are identified (black arrows) x20, b. p16 overexpression in the cytoplasm of the basal cells (black arrows) x40, c. Overexpression of p16 positivity (brown deposits) in the cytoplasm of parabasal cells (stratum spinosum) (black arrows) and koilocytes (blue arrows) x100

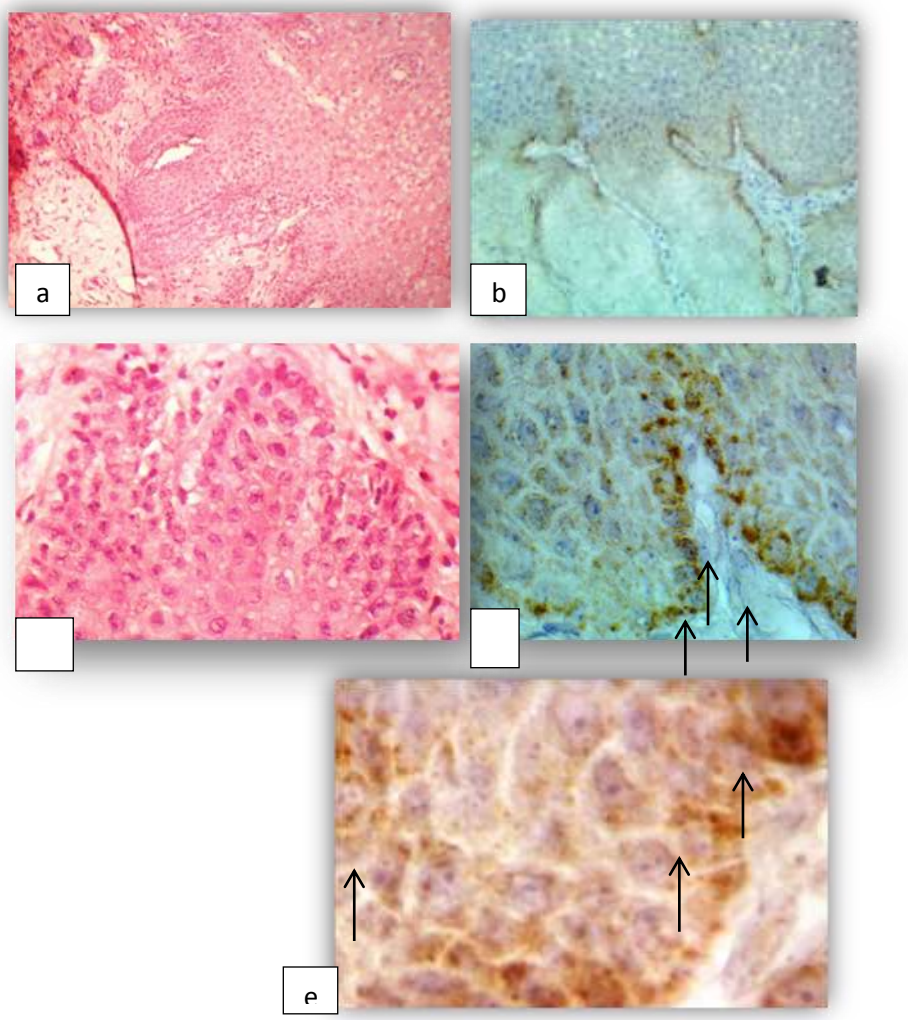

Figure 7: a. H&E stain of cervical biopsy specimen from a 45y old woman Pap smear NILM, histopathology report was (LSIL) x10, b. The same specimen (a) overexpression of p16 in the lower one third of the epithelium x10, c. The same specimen (a) H&E stain X40, d. A moderately positive p16 overexpression in the lower one third (basal and parabasal) of the epithelium (black arrows) x40, e. The same specimen showing p16 overexpression (black arrows) x100

Discussion:

Histopathological evaluation of cervical biopsies from women with abnormal Pap test remains the "gold standard" for the diagnosis and grading of cervical neoplasia [8]. However, diagnosis variability has been documented among observers and depends, in part, on the grade of the abnormality [17]. There is little doubt that the pathology community agrees on the utility of adjunctive stains to increase the accuracy of clinical histologic interpretations [9, 18]. Many studies have provided evidence that p16INK4a immunohistochemistry is valuable adjunctive aid in the diagnosis of difficult cervical biopsies [19, 20, 21, 22].

Murphy and colleagues applied a scoring system based on the percentages of p16 positive cells in the slide [23]. Whereas, Benevolo and associates regarded any nuclear or cytoplasmic reactivity as being positive [24]. In this study we used the semiquantitative scoring described by Klaes and coworkers for the evaluation of this marker on histological specimens [5], this scoring system has been adopted by other investigators [25, 26].

In this study, we investigated the prevalence of p16 expression in a total of 60 women, divided into three groups. Twenty women, with normal looking cervices and normal Pap smears (group I), and 20 women (group II) with unhealthy looking cervices with low and high grade dysplasia with koilocytosis. Group III (20) patients with squamous cell carcinoma SCC. Results obtained demonstrated that none of cervical specimens (group I), evaluated by immunohistochemistry, presented p16 positivity. Whereas, the groupII specimens starting from LSIL (CIN1) to HGSIL (CIN2, CIN3) showed a constant significant increase of protein overexpression. Being only 1 (5%) showed very low positive p16 reaction

(focal areas), 9 (45%) showed moderately positive reactivity, and 10 (50%) with high positive reactivity. Group III demonstrated very high and strong p16 overexpression with stromal invasion.

Our findings corroborate the findings obtained by Benevolo and coworkers , they investigated, p16 expression in 100 cervical biopsies and their obtained results demonstrated that none of the normal cervical tissues, evaluated by immunohistochemistry, presented p16 positivity whereas, starting from CIN1 to CIN2, CIN3 and carcinomas, there was a constant and significant increase of protein overexpression. They concluded that p16 positivity increases with the severity of the lesions and it may be considered as an optional or additional test for precancerous lesions of the cervix [24].

Volgareva and colleagues did not find any p16INK4a-positive cells in control samples and overexpression of p16INK4a was detected in samples of cervical dysplasia (CINs) and carcinomas. These findings are consistent with our findings, but they found that, for all stages of the samples, heterogeneity with respect to p16INK4a expression and p16INK4a-negative CINs and carcinomas did exist. They concluded that p16INK4a-negativity is not a sufficient reason to exclude a patient from the high risk group and p16INK4a may be regarded as a supplementary test for early diagnostics of cervical cancer [10].

Godoy et al in their study of 144 women found that p16INK4 expression was 48.3% in the CIN1/HPV group, as opposed to 94.3% in the CIN2/CIN3 group (P = 0.001), showing a statistically significant difference between the two groups. The reported that the quantitative method they used is simple and less subjective than the semiquantitative method described in the literature and adopted in this study. They concluded that low grade lesions may predispose to progress to high grade lesions. This means that p16INK4 may be a strong marker for "neoplastic lesions" induced by HPV and not just an infection marker [27].

In contrast to our findings, Tringler and coworkers observed and reported that p16INK4a staining was detected in normal cervical specimens. These findings are consistent with Ming Gou and colleagues. In addition, they showed an increase in the expression of p16 protein in accordance to the degree of malignancy of lesions, showing to be a great marker specific for pre-malignant and malignant lesions [28, 29, and 30]. These discrepancies in the results between different groups, including the present study, could be attributed to the arbitrary cutoffs used by different investigators, in addition to the laboratory and personnel constraints that were faced during this study.

Roelens and colleagues concluded that in LSIL triage; p16INK4a can be used as a first-step triage, justifying further diagnostic workup of p16INK4a-positive women. In addition, women with LSIL who are negative for p16INK4a should be reinvited for repeat testing [31].

Galgano on the other hand, found that immunohistochemical staining for p16INK4a is a useful and reliable diagnostic adjunct for distinguishing biopsies with and without CIN2+. He suggested that additional research is needed to identify biomarkers useful in distinguishing CIN from non-CIN [9]. Redman studied whether p16 immunostaining in cervical biopsy could be used for the discrimination of non-HPV-associated lesions from HPV–related ones. She concluded that, p16INK4a can provide an important ancillary technique to pathologists in the setting of possible dysplasia with either confounding metaplastic or reactive histologic changes. This ability to better discriminate between CIN and nonneoplastic equivocal lesions will help reduce false-negative interpretations and improve cervical precancer diagnosis as well as reduce false positive interpretations, thus decreasing unnecessary surgical procedures [32].

In a study conducted by Kalof, 13 of 27 (48%) CIN 1 lesions exhibited diffuse staining with p16INK4a, supporting p16INK4a as a potential marker of dysplasia in difficult lesions (e.g., CIN 1 vs. atypical metaplasia); however, heterogeneous p16INK4a immunoreactivity was reported in CIN 1 lesions, and the usefulness of the marker had not been well established. Because this heterogeneity in staining could represent a difference in aggressiveness among the low-grade lesions, perhaps reflecting the different physical states of the virus (i.e., episomal vs. integrated), it was hypothesized that the low-grade lesions exhibiting higher levels of p16INK4a immunoexpression would show a greater proportion of integrated HPV[33, 34, 28]. These findings are in agreement with Tsoumpou and colleagues who concluded that p16 cannot be used as solitary markers for the assessment of LSIL [35]

On the other hand, Christina Kong evaluated 3 chromogenic *in situ* hybridization (ISH) assays in conjunction with p16INK4a IHC and HPV polymerase chain reaction. She concluded that p16 IHC would be considered as the best candidate for the initial assessment of cervical biopsies that are histologically indeterminate for dysplasia given its wide availability, comparative ease of interpretation, and high sensitivity and specificity [36]. These findings are in agreement with Kate Cuschieri who concluded in her report that there are several properties of p16 that could make this protein a promising biomarker for HPV-related cancers: The expression is directly linked to the HPV oncogene action because continuous expression of E7 is necessary to maintain a malignant phenotype in HPV-associated cancer.

Furthermore, the expression of p16 seems to be independent of the HPV type causing the oncogenic infection, obviating the need to detect

different HPV types in DNA and RNA assays. Also, in contrast to many classic tumor markers, p16 is not associated with proliferation, but rather with senescence and cell cycle arrest, and is not found expressed in normal basal cells or in other cells with proliferative capacity [25]. These conclusions are concordant with the findings of Nicol and many others, she found in her report that the effective sensitivity and specificity of p16INK4a confirms the potential utility of p16INK4a as a diagnostic marker for CIN, particularly in CIN 2-3 and invasive cervical cancer lesions [16].

Conclusion:

In p16INK4a immunohistochemistry IHC would be considered as one of the best candidates for the initial assessment of cervical biopsies that are histologically indeterminate for dysplasia given its wide availability, feasibility and ease of interpretation. These results look promising to be used to triage NILM and LGSIL

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